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For the President of the European Patent Office

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
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Method for histoprocessing

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Title: Method for histoprocessing

The present invention relates to the processing of a biological sample from fixation to impregnation for histological analysis. In particular, it relates to a rapid and safe automated processing system that can be operated with continuous throughput and that eliminates the use of toxic and (in)flammable solvents such as xylene .

Conventional methods for preparing a sample (e.g. tissue) for histology involve incubation in separate solutions of phosphate-buffered 10% formaldehyde for fixation, incubation in a series of increasing concentrations of alcohol for dehydration, and incubation in xylene for clearing tissue of dehydration agent, prior to impregnation. Because of the time required for this process, usually 8 hours or longer, it is customary to complete these separate steps - fixation, dehydration, clearing, and impregnation-overnight in automated mechanical instruments designed for those tasks (see, for example, US3,892,197; US4,141,312; and US5,049,510).

The ultimate goal of tissue processing is to provide the specimen with internal and external support from a medium of like hardness so that the specimen can withstand microtomy without damage. The most common embedding or support medium is paraffin but many other substances are also used. Microtomy is the process of cutting or sectioning an embedded sample or specimen into thin slices of approximately 2 - 8 microns in thickness with a sharp steel knife in a microtome. Slices are then picked up on slides, usually microscope slides.

Standard paraffin processing procedures include exposure to chemical dehydration through graded alcohol solutions, then immersion in a transition solution (commonly referred to as a clearant) followed by

impregnation with paraffin. Dehydration means the removal of water. During processing procedures, dehydration is used to remove the free water molecules and, if performed correctly, also the molecularly bound water. Dehydration is normally accomplished using alcohol solutions; most commonly ethanol, isopropylalcohol (isopropanol); occasionally methanol; or butanol for plant and animal tissue. If specimens are improperly dehydrated and water is left in the specimen, the clearant and impregnation agent (for example paraffin) will not penetrate the tissue and it will be soft and mushy. Excessive dehydration will remove the bound water, causing shrunken, hard, brittle specimens that require excessive rehydration before sectioning.

Fat in a tissue sample is removed with a solvent because fat impairs clearing and impregnation. Inadequate fat removal (defatting) can result in spreading artifacts of tissue sections, wrinkling of tissue sections, and poor staining. Fat may be removed from the tissue specimen with various solvents such as, for example, acetone, chloroform or xylene.

Following dehydration of a sample, a "clearing" agent is used to remove alcohol used for dehydration from the sample and to prepare the sample or specimen for the impregnation medium. Clearing agents, also referred to as "dealcoholization" agents, must be miscible with both the dehydrating agent and the impregnation/embedding medium. Inadequate clearing, which can be caused by water remaining in the specimen or by inadequate exposure times, causes poor paraffin infiltration which will result in soft, mushy specimens. On the other hand, excessive exposure to clearing agents will produce hard, brittle specimens caused by the denaturation of the tissue proteins that is very similar to the effect of excessive dehydration.

Xylene (dimethylbenzene) has been the most widely used clearant for many years. It is an aromatic hydrocarbon that rapidly replaces alcohol and has a refractive index capable of rendering the tissue transparent. A major drawback of xylene is that it is very cumbersome to use, because it is highly volatile, flammable and a suspected carcinogen. Xylene should therefore only

be used with adequate ventilation, and skin contact should be avoided. In addition, xylene is expensive.

Effective replacements for xylene have been actively sought for. A first substitute that was presented in 1981 was limonene. Unfortunately, that chemical has cast a shadow over the subject because of several issues. Limonene is oily and cannot be recycled reliably (the recycled solution is different from the original product). Its odour is overpowering and quickly pervades neighbouring rooms and halls. Most troublesome is the fact that it causes serious sensitization reactions in exposed workers. Other xylene substitutes are short chain aliphatic hydrocarbons (alkanes). Essential oils can also be used as xylene substitute but they are not as common. However, none of these xylene substitutes has proven as useful and cost-effective as xylene .

Conventional procedures for tissue processing can be performed both manually or in an automated fashion. Most histopathology labs now use automated tissue processing machines which use multiple containers and require 6-20 hours for processing. Automated tissue processors implementing such conventional processes are manufactured and sold by, for example, Shandon (HYPERCENTER and PATHCENTRE models), Miles-Sakura (TISSUE-TEK models), and Mopec-Medite (TPC15 model).

A disadvantage of the systems of the prior art is that such automated systems have not been capable of continuous throughput. Given the time required to complete tissue processing, cassettes containing tissues are loaded into the system during the day and tissue processing is completed in an overnight cycle. Thus, operation of the prior art systems did not allow tissue-containing cassettes to be processed to completion during the work day.

Typically such conventional methodology demands sending tissue specimens from the operating room, medical office or other sites, to a pathology laboratory sometime during the working day, followed by overnight batch processing of the specimens, so that a tissue specimen suitable for blocking and sectioning is at the earliest only available on the morning of the next day;

and rendering a diagnosis by a pathologist based on microscopic examination of sections prepared from a blocked and sectioned specimen is only possible later on that next day. This minimally requires almost 24 hours between receipt of the specimen and delivery of the pathologist's report.

In addition to the minimum one-day delay in giving a medical practitioner (e.g. a surgeon) the benefit of a report from the pathologist, there are also problems associated with impeded work flow in the pathology laboratory necessitated by the requisite batch processing of specimens, the safety concerns that attend having instruments operating overnight, the risk of possible instrument failures and the need to monitor the instruments, and the waste of using large volumes of reagents for such processing when automated. Moreover, expensive measures are required to prevent exposure of laboratory personnel to noxious fumes and toxic substances associated with the reagents (such as xylene) used in this process. Also, the large volumes of solvent waste and paraffin debris produced by the conventional methodology will pollute the environment if not properly disposed.

There is an ever present interest in expediting tissue processing and analysis for diagnostic purposes. Furthermore, recent healthcare focus has been directed to lessening the cost of various procedures including tissue processing. The costs of tissue processing are related to the time for processing and analysis of the specimens, the space required for the personnel and equipment in the laboratory, the volume of reagents (both the purchase price of the pure chemicals and the charges for discarding waste), and the number of personnel required. More importantly, patients and their physicians depend on evaluation and diagnosis by the pathologist to guide treatment. Reducing the amount of time needed to complete tissue processing would lessen the anxiety experienced during the period between obtaining the specimen and delivering the pathologist's report to the physician. Thus, a significant reduction in the time required for processing of a histological sample is very desirable. Others have also recognized the need to shorten the time required for tissue

processing, but they have made only modest improvements in the conventional methods. To accelerate tissue processing, U.S. Pat. Nos. 4,656,047, 4,839,194, and 5,244,787 use microwave energy; U.S. Pat. Nos. 3,961,097 and 5,089,288 use ultrasonic energy; and U.S. Pat. No. 5,023,187 uses infrared energy. U.S. Pat. No. 5,104,640 disclosed a non-aqueous composition of a fixative, a stabilizing agent, and a solubilizing agent that adheres a blood smear to a slide.

The inventors have now recognized that a supercritical fluid may advantageously be used in histoprocessing. The invention describes a method for tissue processing in a manner distinct from any of the procedures currently used. Provided is a method for processing a biological sample for histological (or pathological) analysis, characterized in that the sample is contacted with a composition comprising a supercritical fluid. A method in accordance with the present invention is faster than any of the reported methods, causes minimal damage to the processed tissue, avoids the use of organic solvents including xylene and, surprisingly, results in a superior specimen for subsequent cytological, histological or anatomical analysis. In addition, with a method according to the invention it is no longer necessary to use large (plastic) containers with an inflammable liquid such as ethanol or xylene.

A supercritical fluid, sometimes called a supercritical gas fluid or a fluidum, is any substance above its supercritical temperature and supercritical pressure. For every substance there is a temperature above which it can no longer exist as a liquid, no matter how much pressure is applied. Likewise, there is a pressure above which the substance can no longer exist as a gas no matter how high the temperature is raised. This point is called the supercritical point; the critical temperature and critical pressure are the defining boundaries on a phase diagram for a pure substance.

In the supercritical area there is only one state-of-the-fluid. A supercritical fluid exhibits physicochemical properties intermediate between those of liquids and gases. Supercritical fluids (also known as highly condensed gases) are able to spread out along a surface more easily than a true liquid because they have lower surface tensions than liquids. At the same time, a supercritical fluid maintains a liquid's ability to dissolve substances that are soluble in the compounds, which a gas cannot do. The phenomena of enhanced solubilities in supercritical fluids has been known since the late 1800s. For decades they have been used in food processing industries to extract flavoring compounds such as caffeine and hop oil. The solubilizing power of supercritical fluids is sensitive to small changes in the operating conditions, and it is possible to fine-tune the pressure and the temperature to tailor the solvent capacity of a supercritical fluid for a particular process. Their desirable and unique properties have provided the impetus for applying supercritical fluid technology to various other problems, e.g. the cleaning of fabrics or the sanitization of contaminated soil. The present invention is the first to show that the unique characteristics of supercritical fluids can advantageously be exploited in the field of histological sample processing. It may not always be necessary to use a substance that is at or above its supercritical point, as long as the properties (especially the solubilizing capacity) of the substance are of use in a method for histoprocessing. For example, a subcritical fluid which is at a pressure and temperature just below its supercritical point can also be advantageously used in a method provided herein.

The invention provides a method of processing a biological sample for histological analysis, characterized in that the sample is contacted with a composition comprising a supercritical fluid (or a substance with a solubilizing capacity similar to that of a supercritical fluid). According to the invention, a (tissue) sample is contacted with or surrounded by a supercritical fluid wherein contacting the sample comprises pressurizing the sample with the

composition comprising a supercritical fluid to above the critical pressure of the supercritical fluid and heating the sample with the supercritical fluid to above the critical temperature of the supercritical fluid. The supercritical fluid penetrates a sample as it passes through a sample in a high-pressure vessel. In a preferred embodiment, a sample is contacted with a composition comprising supercritical carbon dioxide (CO_2). The supercritical pressure of CO_2 is about 7,3 MPa (73 bar) and the supercritical temperature is approximately 31 degrees Celsius. Biological tissues contain proteins which denature by temperature above approximately 60 °C. The relatively low supercritical temperature of CO_2 allows to contact a sample with a supercritical fluid at a temperature that has essentially no detrimental effects on the biological sample. However, other supercritical fluids with a relatively low supercritical temperature (preferably lower than 60 °C) are also suitable for use in a method according to the invention, for example xenon, nitrous oxide, ethane, HFC-116, chlorotrifluormethane, ethylene, sulfur hexafluoride and trifluormethane.

Carbon dioxide is extremely attractive in industrial applications because it is the second most abundant and the second least expensive solvent on earth. It is non-flammable, non-toxic, readily available in high purity.

The invention provides a method for processing a biological sample for histological analysis characterized in that the sample is contacted with a composition comprising a supercritical fluid, wherein said processing comprises dehydration, defatting, decalcification and/or impregnation of said sample. In one embodiment, a method is provided for processing a sample wherein said processing comprises removing at least one substance from said sample. For example, in one embodiment a sample is contacted with a supercritical fluid to solubilize and remove water from said sample. A supercritical fluid can also be mixed with an other solvents, to aid in extracting certain substances from a sample. In one embodiment, a sample is contacted with a composition comprising a supercritical fluid, wherein said

composition additionally comprises a dehydrating agent, preferably an alcohol, such as ethylalcohol (ethanol; EtOH) or a detergent, such as Tween. Dehydration using a supercritical fluid as provided herein is typically accomplished fast, sometimes even within minutes. Thus, the invention provides an attractive alternative for the time-consuming traditional step-wise dehydration using graded alcohol solutions. Advantageously, the supercritical fluid dissolves and extracts other substances from a sample, such as fats and lipids, thereby facilitating cutting a sample into thin slices. In a specific embodiment, processing of a sample, in particular a calcified tissue like a bone specimen, comprises removing calcium from a sample. Decalcification of a sample is important for cutting thin slices from bone tissues and other calcified particle in tissues, because calcified structures are in general difficult to cut. Traditional decalcifying protocols require the additional incubation of a fixed sample during one to five nights in an acidic decalcifying solution (typically formic acid, acetic acid, hydrochloric acid or nitric acid). Decalcification is also performed using a calcium-chelator such as EDTA (ethylenediaminetetra acetic acid). According to the invention, decalcification of a sample is accomplished simpler and faster when compared to existing decalcification methods. Hereto, a biological sample is contacted with a composition comprising a supercritical fluid, wherein said composition additionally comprises a decalcifying agent. Suitable decalcifying agents include acids such as carboxylic acids, for instance formic acid or acetic acid, and other chemicals capable of binding or sequestering calcium.

When a composition comprising a supercritical fluid and additionally comprising a co-solvent (e.g. for water and/or calcium) is used, the supercritical fluid and the co-solvent (e.g. an alcohol and/or an acid) can be supplied as a mixture in a cylinder. However, the concentration of the co-solvent in a supercritical fluid is not constant when the cylinder is emptied, and thus more stable results can be achieved by using pump systems onsite to mix the required volume of co-solvent.

In a method in accordance with the present invention, the use of a supercritical fluid is not limited to extracting a substance from a sample into the fluid. In contrast, a supercritical fluid is also advantageously used to provide a sample with a substance dissolved in said fluid. In a preferred embodiment, a sample is impregnated with a composition comprising a supercritical fluid, wherein said composition additionally comprises an embedding medium, preferably a liquid embedding medium such as liquid paraffin. Such a solution of an embedding medium in a supercritical fluid according to the invention is also referred to as impregnation fluid. Following passage of the impregnation fluid through a sample above the critical pressure and critical temperature of the supercritical fluid, the temperature and / or pressure are successively lowered such that the fluid is no longer in the supercritical state and has lost its unique solvent properties. This causes the dissolved substance (herein: embedding medium) to be released from the fluid into the sample. In a preferred embodiment, impregnation according to the invention using a impregnation fluid directly follows dehydration according to the invention, yielding a sample wherein the water is quickly replaced by an embedding medium without the use of traditional transition solutions or clearants. An embedded sample of the invention can be used for further processing by standard technologies used in the field.

Other preferred embedding or impregnation materials are commercial wax formulae, mixtures of waxes of different melting points (e. g., liquid mineral oil and solid paraffin), paraplast, bioloid, embedol, plastics, and the like. Paraffin has been chosen for use in the examples herein because it is inexpensive, easy to handle, and ribbon sectioning is facilitated by the coherence of structures provided by this material. A supercritical fluid, preferably CO_2 , may for instance be mixed with liquid paraffin that is heated to 55 °C. If the paraffin is mixed with CO2 , the melting point of paraffin is reduced.

Histological analysis as used herein refers to any type of analysis that may be performed to study the appearance, properties and behaviour of a tissue, a cell, an organ or an organism. It can be carried out by inspecting a processed sample under a microscope. A (component of a) processed sample can be contacted with one or more reagents, such as a dye, a reagent or a probe specifically reactive with one or more components (such as proteins, nucleic acids, carbohydrates) present in the sample to identify or mark a certain cell type or tissue. A tissue refers to a group or layer of cells which are essentially alike and work together to perform a specific function. Typical probes include antibodies (e.g. for immunohistochemistry), nucleic acid (RNA; DNA) probes (e.g. for in situ hybridisation or PCR techniques), substrates for use in enzyme histochemistry (e.g. NADH for detecting acetyl cholinesterase or ATP-ase activity) and conventional staining chemicals such as hematoxylin and eosin (H&E); Alcian Blue for sulfated mucosubstances ; Brown-Brenn Gram stain for Gram-positive and Gram – negative bacteria; Congo Red for amyloid; Giemsa for *H. pylori*; and Bone Marrow; Gomori's Modified Iron stain; and many other probes known to a person skilled in the art that are of use to identify or mark a certain cell type or tissue, be it normal or diseased.

Pathological analysis refers to histological analysis that is related to pathology. Typically, pathological analysis is performed by a pathologist who diagnoses disease by studying a sample comprising cells and tissues under a microscope. In a preferred embodiment, a pathological analysis comprises analysis of a human sample to stage or grade a disease.

According to the invention, a sample comprises a biological sample, such as a tissue specimen. In the context of the invention, a "tissue specimen" is any piece of tissue that may be processed by a method disclosed herein. It may also refer to single cells from any biological fluid (e. g., ascites, blood, pleural exudate), or a cell suspension obtained from aspiration of solid organs or lavage of body cavities. Single cells may be pelleted by sedimentation or buoyant centrifugation prior to processing. It may also refer to an intact organ,

or even an intact organism, or a part thereof. Organisms include unicellular and multicellular organisms, and range from bacteria, fungi, insects and plants to mammals. Solid pieces (i. e., tissue slices or needle biopsies) from a human subject are commonly processed for histology and pathology. Where conventional methods for the fixation and embedding of an organ (e.g. brain) require up to 6-8 weeks, a method of the invention now allows impregnation of an (intact) organ or a part thereof with a solid embedding medium within a day and without the use of toxic (clearant) solvents.

With a method of the invention, it is possible to reduce total processing time (from fixation to impregnation) from the conventional 8-12 hours to less than 2 hours, preferably less than 1.5 hours, more preferred less than one hour. Nowhere in the prior art it has been taught or suggested that the entire process of preparing diagnostic tissue slides could be accomplished in less than 1.5-2 hours, starting from the preparation of a specimen from a fixed or non-fixed tissue and ending with impregnation, with continuous processing of specimens and circumventing the use of toxic, possibly carcinogenic clearants. WO 01/44783 discloses a tissue processor system including an improved microwave unit that allows rapid processing under two hours and, optionally, without the use of xylene clearants. However, the protocol of WO 01/44783 can only process a tissue specimen of less than about three millimetres. In contrast, in a method of the present invention, samples up to 5 millimeters, such as 8 mm or even more than 1 centimeter may be rapidly processed using a supercritical fluid. As mentioned above, and unlike WO 01/44783, a method of the invention is not limited to tissue samples or small tissue sections. A method according to the invention allows to process a sample with a volume of up to 0,001 cm^3 (e.g. a biopsy), preferably up to 1.0 cm^3 (e.g. a skin specimen), more preferred even up to 10 cm^3 (e.g. a small tumor), most preferred up to 2000 cm3 (e.g. an organ such as complete brains). In general, according to a method of the invention, the larger the sample, the more time it will take to process the sample. For example, a tissue specimen of

20 x 15 x 5 mm is rapidly dehydrated and impregnated according to a method of the invention. This offers a considerable advantage, in that a sample can be processed of such a volume or size that it is possible to obtain multiple (microtome) slices from said sample. For instance, following histological inspection of a sample, a pathologist may want to inspect the same sample that has been stained with a specific reagent, such as an antibody, to aid in the histological analysis. Processing a sample according to the invention allows to simply provide a second, third or even higher number of (parallel) slices from the same sample. If the specimen is already less than 3 millimeter before processing, as is the case in WO 01/44783, this is obviously not possible. Instead, multiple samples need to be taken at the outset and their relative orientation needs to be carefully registered to reconstruct their connection *in situ*.

Histological analysis of various types of human tissue specimens that were processed using a supercritical fluid according to the invention surprisingly revealed a superior quality of the specimen compared to specimens from the same tissue sample that were processed according to conventional procedure using ethanol as a dehydration agent and xylene as a clearant. For example, the keratin staining pattern of a human colon specimen following processing using carbon dioxide shown in Figure 3 is more intense compared to the keratin staining pattern of Figure 2, showing a specimen from the same colon sample which was processed using conventional methods. Likewise, improved histological analysis could be performed on a vimentin-stained human gall bladder specimen processed according to a method of the invention (compare Figures 3 and 4) and on a human nerve specimen stained for S-100 protein (compare Figures 6 and 7).

In one embodiment of the invention, a sample is fixed according to conventional methods prior to being processed according to a method according to the invention, for instance using a formaldehyde solution (also known as

formalin). Formaldehyde (CH_2O) reacts with terminal free -NH_2 groups of proteins and forms covalent methylene bridges between two components of a protein or between two different proteins. However, a major drawback of conventional fixation and tissue processing (i.e. into paraffin blocks) lies in the fact that it can cause irreversible damage (e.g., hydrolysis of a phosphodiester bond and/or deamidation) to the structure of nucleic acids (e.g., DNA, and especially RNA). Accordingly, fixing and processing of a (tissue) sample into a paraffin blocks limits the application of genetic techniques for diagnosis and research.

In another embodiment, a sample that has been previously frozen is processed according to a method of the invention. It is known in the field that most DNA and certainly RNA analyses require special precautions with handling of sample material, such as immediate ("snap") freezing of fresh tissues into liquid nitrogen to prevent nucleic acid degradation. A method of the present invention can be used for processing a (snap) frozen sample and the invention thus provides a method for obtaining a processed biological sample that can subsequently be analysed for various types of histological analysis, including nucleic acid (DNA, RNA) analysis. On the other hand however, histological diagnosis of a frozen section may suffer from disadvantages in comparison to sections prepared from paraffin blocks. For instance, frozen sample are prone to dehydration. Storage of frozen samples therefore requires measures to prevent dehydration. Importantly, frozen tissues often show many artefacts that are caused by the presence of ice crystals in the sample. Thus, it may sometimes be difficult to adequately weigh the advantages and disadvantages that are associated with either a fixed or frozen samples against each other.

A method in accordance with the invention for processing a sample now provides an elegant solution to these problems, because in addition to processing a fixed or frozen sample, it also allows for processing a fresh sample that has not been fixed or frozen prior to being contacted with a supercritical

fluid. Instead of freezing or using a chemical fixative, the high pressure encountered by the sample during contacting with a supercritical fluid and the rapid impregnation with a solid embedding medium (paraffin wax) ensures an optimal preservation of the structure and architecture. Surprisingly, no damage to the tissue sample occurs if pressure is gradually increased and subsequently decreased. Thus, the invention provides an attractive alternative for snap freezing a sample and allows for preparing a sample that is compatible with multiple types of (pathological) analysis including histological, biochemical and nucleic acid analysis, without the use of formalin.

Importantly, in addition to the reduction in time required for tissue processing, the rapid tissue preparation using a supercritical fluid allows for preserving tissue structures and morphology that are lost with conventional methodology. Glycogen, which is an important compound giving strength to biological structures, is almost always lost using the conventional methodology. Lymphatic vessels, particularly of the myometrium, collapse during conventional processing while they remain essentially intact when a method of the present invention is used. Moreover, studies with tissues processed in accordance with the invention indicate better preservation of DNA and RNA extraction as compared to conventional processing methods. Tissues obtained in hospitals and other surgical settings can be processed for both histological and genetic studies soon after delivery to the laboratory. In addition, because a sample processed in accordance with the invention is typically well preserved, archival sample material may be made available for future research and other applications.

The present invention does not prohibit preparation of nucleic acids, DNA or RNA, from processed samples. Thus, genetic study is possible for specimens collected routinely in the clinical pathology laboratory. The combined power of these technologies will be great. Histological observations may be correlated with results from genetic studies by analyzing one histochemical section by staining or immunohistochemistry, and analyzing

nucleic acids from an adjacent section for genetic analysis (using for example PCR techniques). For example, diseased and normal regions of the same section may be compared to detect genetic differences (e. g., mutations, levels of transcription), disease progression may be characterized by comparing genetic differences in samples taken at several time points, and tumor evolution may be assessed by following the accumulation of genetic differences from primary cancer to metastasis.

A further advantage of a method according to the invention relates to sample or specimen orientation. Specimen orientation is key to reach the end result - the correct diagnosis. In existing procedures, a processed (embedded) specimen is placed in a specimen holder or mold to enable sectioning of the tissue in a microtome. Fixation and immobilization of a sample, frequently small and delicate, in the correct orientation into a holder is often troublesome. Because most glues are not compatible with the organic solvents used in histoprocessing, molds with a roughened or "sticky" surface are typically used to attach a specimen to the bottom of a mold or holder. However, these holders often do not sufficiently fix a specimen such that it can be sectioned. Other specimen holders are provided with snap-on lids to immobilize a sample by simply clamping a sample in between the bottom and the lid. Still, these holders are not suitable for delicate specimens such as skin or epithelial tissues because the pressure required to clamp a specimen easily disrupts the integrity of such tissues, e.g. evidenced by collapsed blood vessels.
The invention now provides a solution to these problems. Since a method of the invention does no longer require organic solvents (xylenes) which previously disqualified the use of glues, a specimen can now simply be glued in a desired orientation to the bottom of a holder. Moreover, as mentioned earlier, a method of the invention is suitably used to impregnate samples of considerably larger size than could be used thus far. As a consequence, it is now even possible to first orient an intact tissue, organ or even organism, prior to processing. For example, a tumour is removed in the operating theatre. Instead of cutting the

tumour into multiple small size specimens, thereby carefully keeping track of their relative orientation, the invention now permits to orient and embed the whole, intact tumour.

We have developed a simple, safe, low cost, expeditious, and reliable process that permits preparation of impregnated tissue blocks suitable for microtome sectioning in less than 1.5 hours from the moment tissue is received in the pathology laboratory. The invention allows continuous processing and flow of specimens, either fresh, fixed or frozen, is adaptable to automation, precludes the need for formalin and xylene with their noxious fumes, allows standardization of tissue processing, and requires considerably smaller volumes of reagents than conventional methods. By "continuous" processing, we mean accessing the system of the invention with additional tissue specimens at intervals determined by the time required to complete an individual step of the process (i.e., a few minutes) instead of the time required to complete the process (i.e., an hour to several hours). At any given time, there can be samples at different stages of processing. In other words, a continuous throughput and flow of specimens along the various stages of tissue processing is made possible by the invention. Continuous processing may be accomplished manually or by an automated instrument, such as a tissue processor.

In one aspect of the invention, a processor is provided for use in a method of the invention. An embodiment of such a processor according to the present invention will now be described by way of example with reference to Figure 1 in the accompanying drawing.

Figure 1 schematically shows an example of a processor according to the invention.

The processor 1 of Figure 1 comprises a storage tank 3 and a conduit system 4. The conduit system 4 has an inlet 2 for supplying to the processor a substance, in this example assumed to be liquid carbon dioxide. In use carbon dioxide is transported through a conduit of the conduit system 4 to the storage

tank 3. The processor 1 comprises downstream of the storage tank 3 pressurizing means 5 and heating means 6, both for bringing the carbon dioxide in supercritical phase. Further downstream, the processor 1 comprises two pressure vessels 7 and 8 in which the carbon dioxide, possibly intermixed with co-solvents, is maintained in supercritical phase. The pressure vessel 7 for instance in use may contain supercritical carbon dioxide intermixed with an alcohol, whereas pressure vessel 8 may contain supercritical carbon dioxide intermixed with paraffin. The supply of co-solvents to the pressure tanks and the mixing of substances can be performed in many different known ways.

The mixtures in the pressure vessels 7 and 8 can be supplied to a process reactor 9. This reactor 9 may contain samples 10 to be prepared by the processor 1 for histological analysis. The reactor 9 comprises pressurizing and/or heating means 14 for maintaining the carbon dioxide in the reactor 9 in supercritical phase. With these conditioning means 14 of the reactor 9 the different steps of the different methods of the invention as described above can be performed in the process reactor 9. Downstream of the process reactor 9 the processor 1 further comprises extraction means 11 for extracting different substances from the mixture of substances leaving the reactor 9. The extracted substances such as alcohol, paraffin, water, etcetera, can leave the extraction means through outlets 12. Some of these substances, for example paraffin, can be re-used. The carbon dioxide left over from the mixture can be recycled. To that end, it is treated in a carbon dioxide gas condensator 13 comprised in the conduit system 4 of the processor 1, after which the liquefied carbon dioxide is fed to the storage tank 3 again.

Having described an example of a processor 1 according to the invention, many modifications thereto will become apparent to those skilled in the art without deviation from the invention as defined by the scope of the appended claims. For example it is possible to apply in a single processor a larger number of reactors, pressure vessels, storage tanks, etcetera.

LEGENDS

Figure 1

Example of a processor according to the invention.

Figure 2

Human colon specimen (100x; insert 400x) processed according to the conventional high throughput Sakura VIP-300 bench-top automated tissue processor. Keratine was stained using Keratine 20 (monoclonal Mouse Anti Human Cytokeratin 20 Clone K_s 20.8 clone no M7019 lot 067 from Dako). A Ventana NexES™ automated immunostainer was used.
Specificity: epithelial cells of the mucosa of the large intestine are stained, no background staining visible.
Intensity: diffuse staining pattern along the entire crypt.

Figure 3

Human Colon specimen (100x; insert 400x) from the same sample as shown in Fig.2 but processed using super critical carbon dioxide. Staining was performed as described for Figure 2.
Specificity: epithelial cells in the mucosa of the large intestine are stained, no background staining visible.
Intensity: diffuse staining along the entire crypt, staining is enhanced compared with the conventional method (VIP).

Figure 4

Human gall bladder specimen (100x; insert 400x) processed by standard procedure VIP300.
Vimentin was stained using an anti-vimentin antibody (clone vim 3B4) cat nr 112457 Boehringer Mannheim) and the Ventana staining according to protocol.

Specificity: mesenchymal cells in the lamina propria and the deeper layers of the gallbladder are stained. Epithelial cells are negative.
Intensity: diffuse intracytoplasmatic staining of the mesenchymal cells.

Figure 5

Human gall bladder specimen (100x; insert 400x) of the same sample as shown in Fig. 4 but processed using supercritical carbon dioxide.
Staining was performed as described for Fig. 4.
Specificity: mesenchymal cells are stained in the lamina propria and deeper layers of the gall bladder.
Intensity: enhanced compared with the conventional procedure (VIP).

Figure 6

Human nerve specimen (400x) processed using the standard procedure VIP300. Staining was performed using antibody: S-100 Code no.Z 311 lotno. 026 Dako. Ventana staining according to protocol
Specificity: nerves are stained and other structures are negative.
Intensity: diffuse intracytoplasmatic staining of the Schwann cells and the neurons. No background staining visible.

Figure 7

Human nerve specimen (400x) of the same sample as shown in Fig. 6 but processed using supercritical carbon dioxide.
Staining was performed as described for Fig. 6.
Specificity : nerves are stained and other structures are negative.
Intensity: strong staining of the Schwann cells and the neurons. No background staining visible.

Claims

1. A method for processing a biological sample for histological analysis, characterized in that the sample is contacted with a composition comprising a supercritical fluid.

2. A method according to claim 1, wherein said supercritical fluid is supercritical carbon dioxide.

3. A method according to claim 1 or 2, wherein said biological sample is a fresh, frozen or fixed tissue sample.

4. A method according to any one of claims 1-3, wherein said biological sample comprises an organ or a part thereof.

5. A method according to any one of claims 1-4, wherein said processing comprises dehydration, defatting, decalcification and/or impregnation of said sample.

6. A method according to any of the preceding claims, wherein said composition additionally comprises a dehydrating agent, preferably an alcohol.

7. A method according to any of the preceding claims, wherein said composition additionally comprises a decalcifying agent, preferably an acid.

8. A method according to any of the preceding claims, wherein said composition additionally comprises an embedding medium, preferably paraffin.

9. A sample for histological analysis, characterized in that said sample is processed using a supercritical fluid.

10. A sample according to claim 9, wherein said sample is processed starting from a fresh, non-fixed sample.

11. Use of a supercritical fluid for processing a sample for histological analysis.

12. A processor (1) for preparing at least one sample (10) for histological analysis, comprising at least one process reactor (9) for the at least one sample (10), characterized in that the processor (1) comprises supplying means (4) for supplying to the reactor (9) at least one substance of which at least one is in supercritical phase, and that the reactor (9) comprises pressurizing and/or heating means (14) for maintaining at least one substance in supercritical phase.

13. A processor (1) according to claim 12, further comprising at least one pressure vessel (7, 8) for mixing different substances and for maintaining at least one substance in supercritical phase.

14. A processor (1) according to claim 12 or 13, further comprising pressurizing and/or heating means (5, 6) for bringing a substance in supercritical phase.

15. A processor (1) according to any one of the claims 12-14, further comprising extracting means (11, 12) for extracting substances from a mixture of substances.

16. A processor (1) according to any one of the claims 12-15, further comprising recycling means (13) for recycling substances discharged from the reactor (9).

17. Use of processor according to any one of the claims 12-16 for processing a biological sample for histological analysis.

Title: Method for histoprocessing

Abstract

The invention relates to the processing of a biological sample for histological analysis. In particular, it relates to a rapid automated processing system that can be operated with continuous throughput and that eliminates the use of toxic solvents such as xylene. Provided is a method of processing a biological sample for histological analysis, characterized in that the sample is contacted with a composition comprising a supercritical fluid. Also provided is a processor (1) for preparing at least one sample (10) for histological analysis, comprising at least one process reactor (9) for the at least one sample (10), characterized in that the processor (1) comprises supplying means (4) for supplying to the reactor (9) at least one substance of which at least one is in supercritical phase, and that the reactor (9) comprises pressurizing and/or heating means (14) for maintaining at least one substance in supercritical phase.

Fig. 1

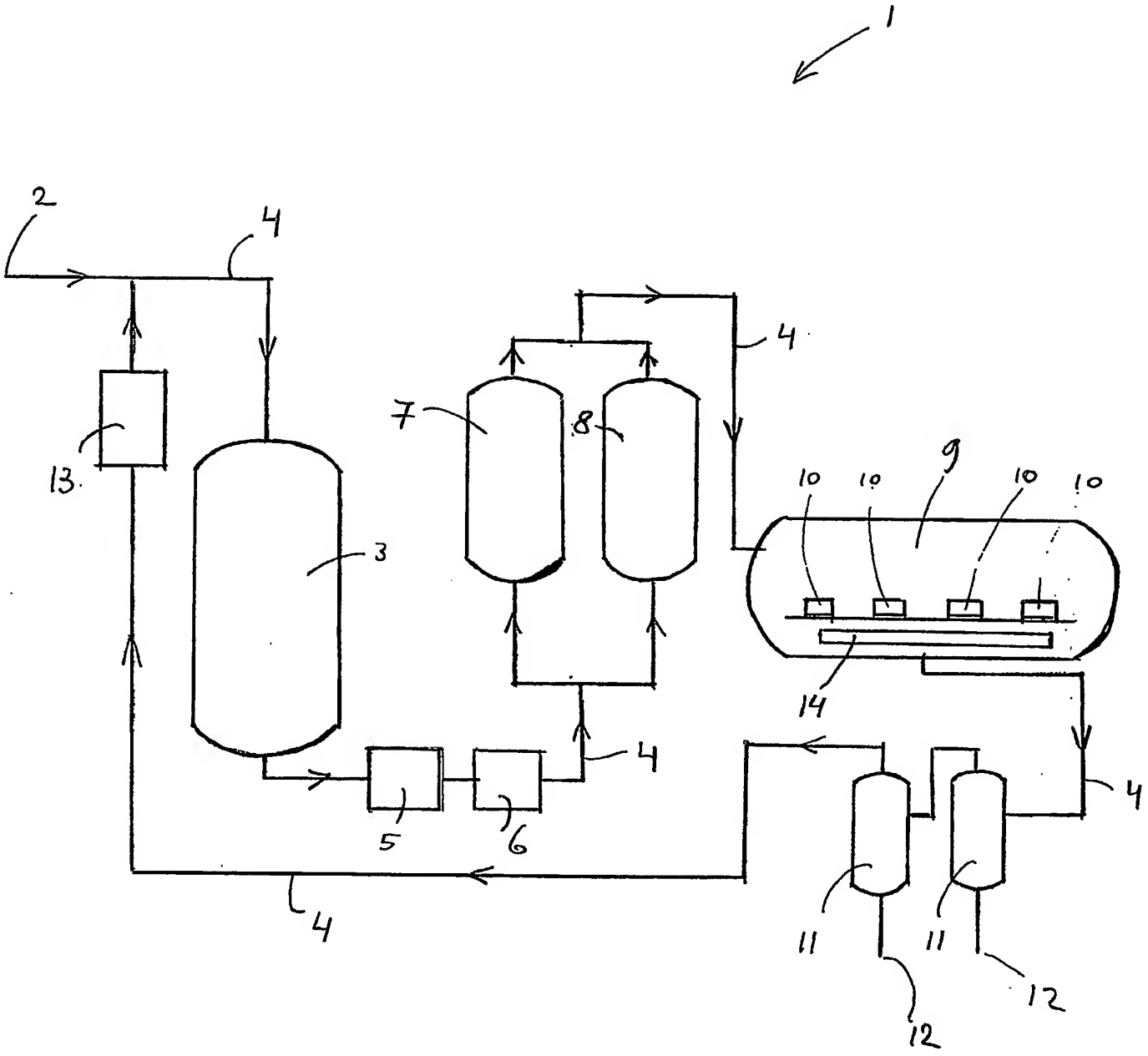

Figure 2

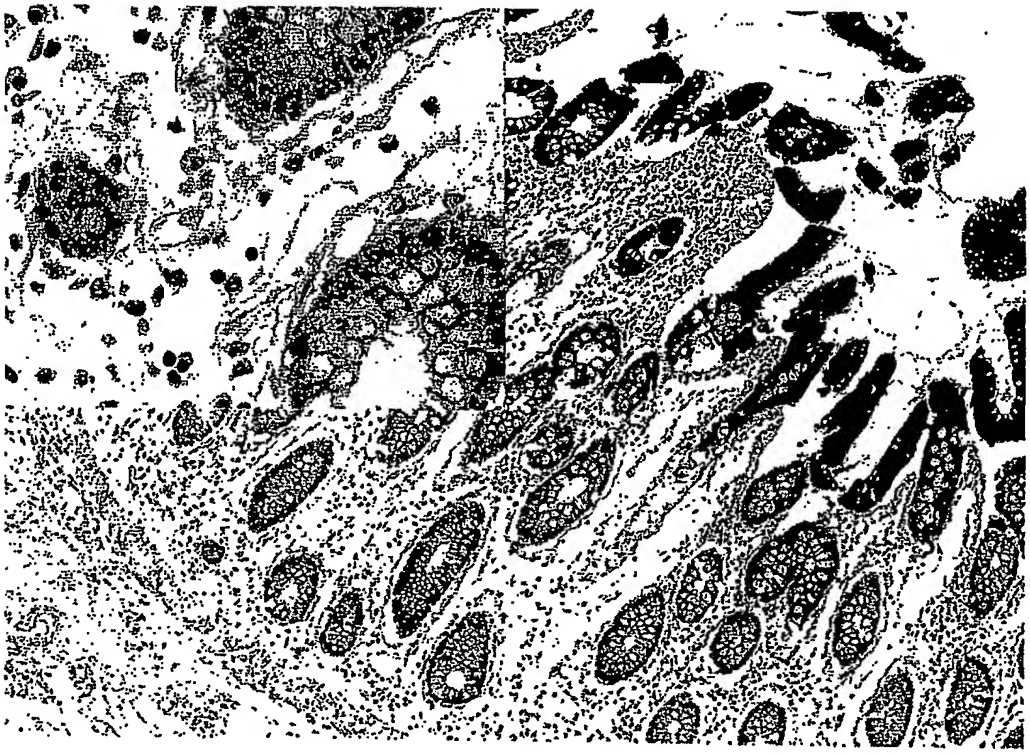

Figure 3

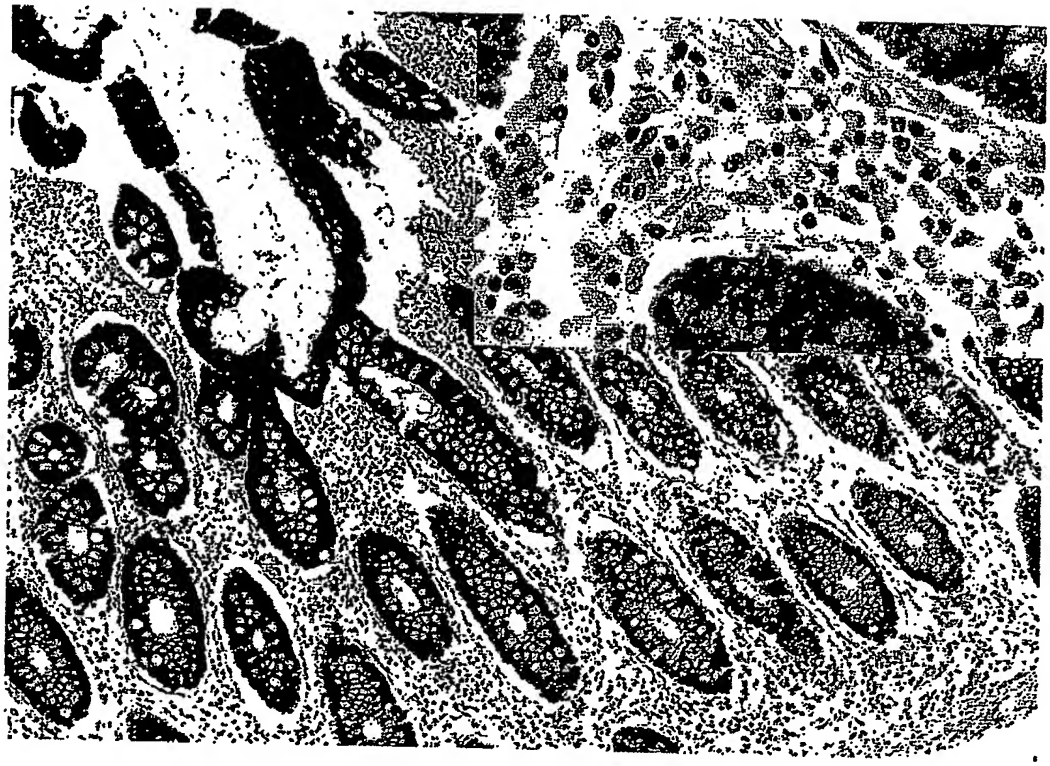

Figure 4

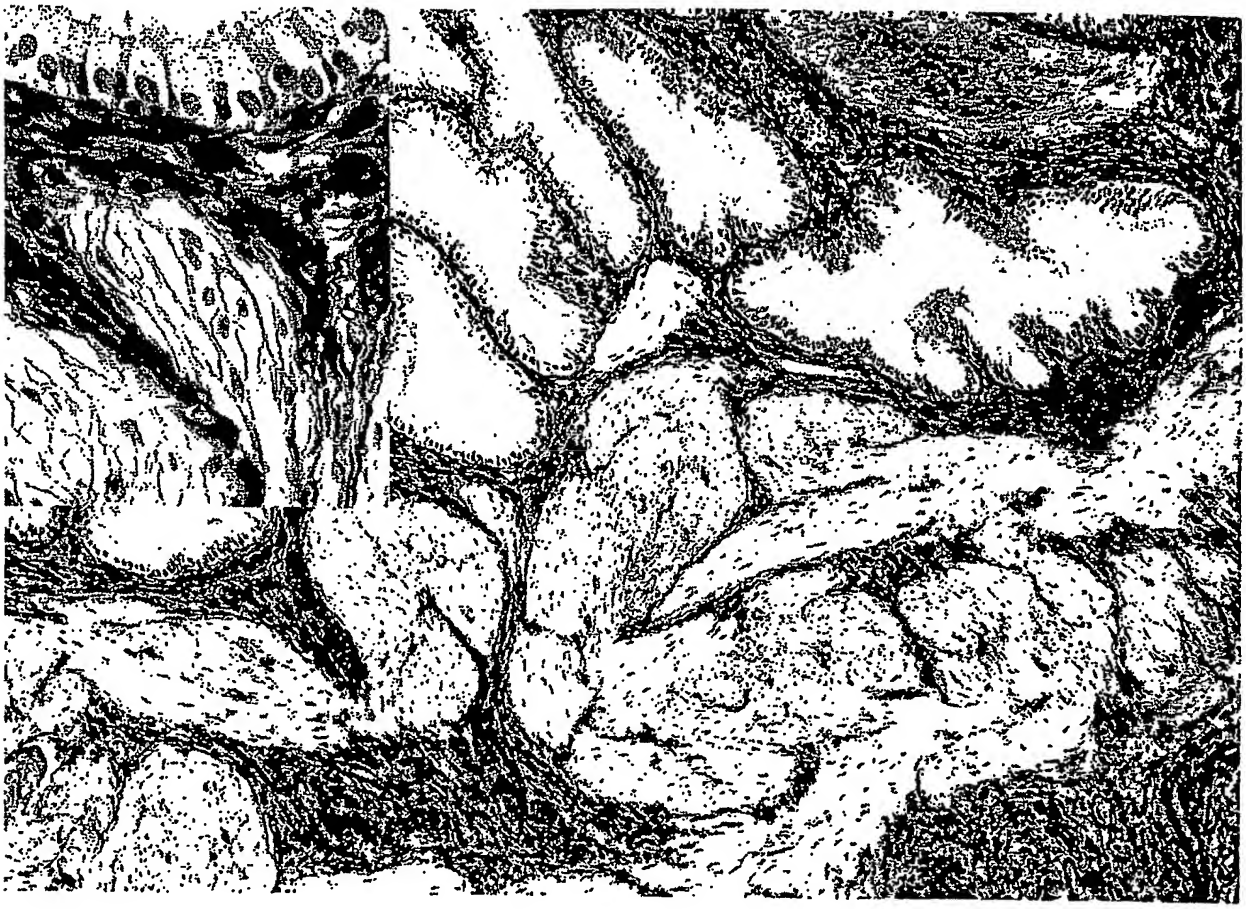

Figure 5

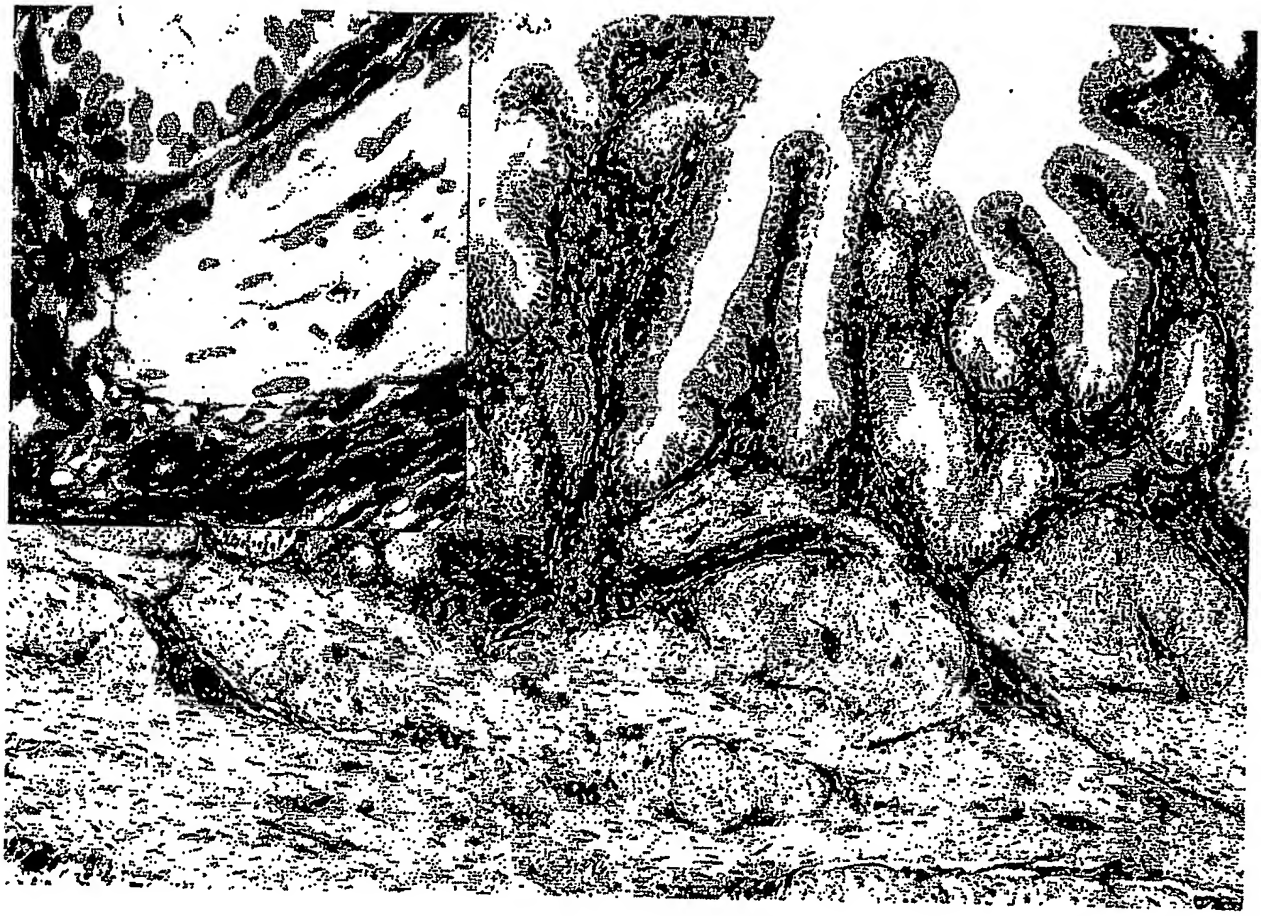

Figure 6

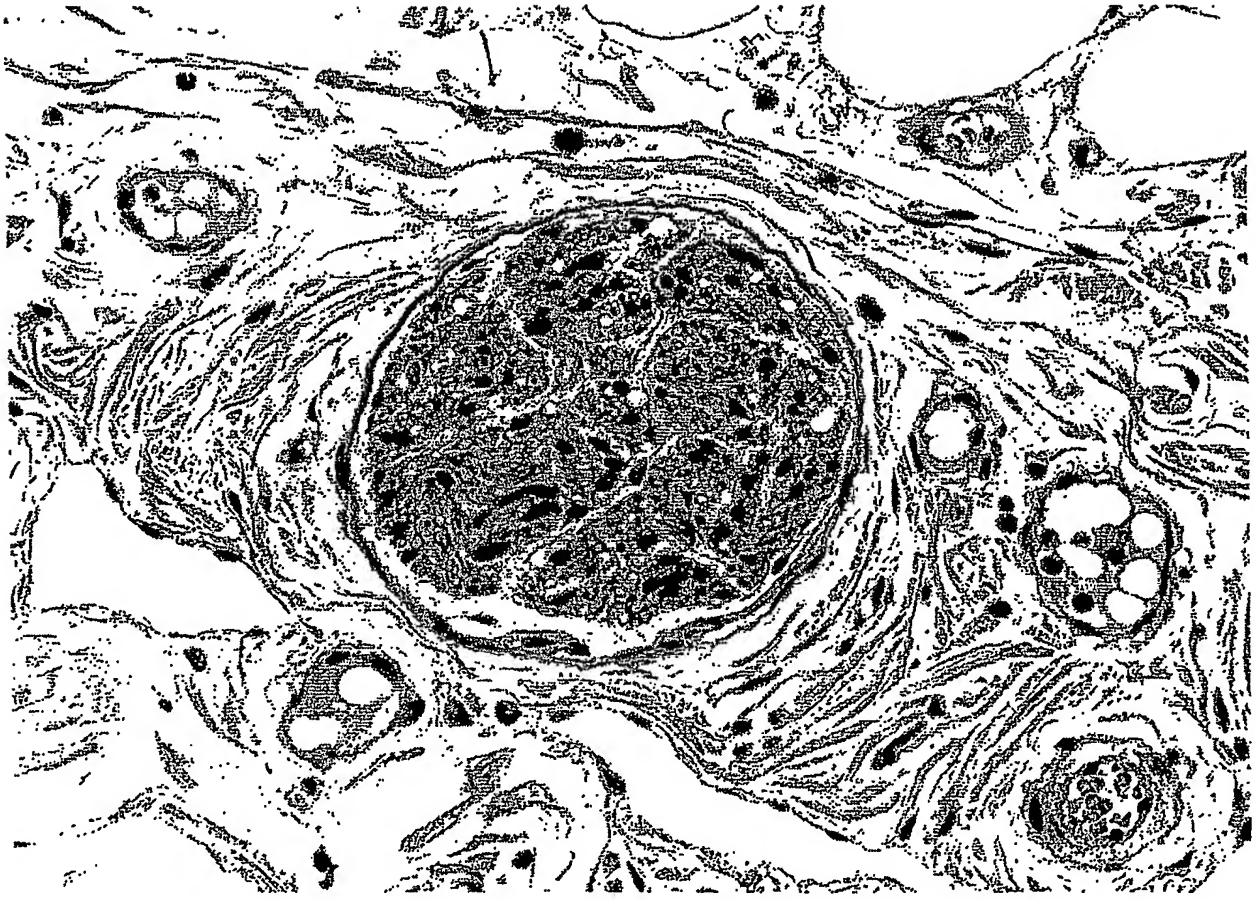

Figure 7